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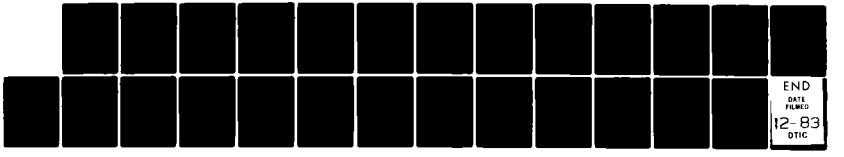

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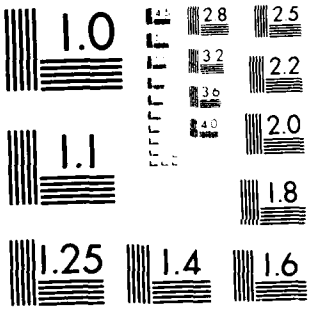

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THE ROLE OF INTESTINAL BACTERIA IN ACUTE DIARRHEAL DISEASE

Annual and Final Report

Sherwood L. Gorbach, M.D.

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Our studies provide evidence for at least 3 serologically distinct groups of surface antigens present on ETEC isolated from man. E. coli strains in each of the 3 serologic groups produce surface pili of identical size, mophology, and molecular weights. Antisera against these three pili types were used to test 106 strains of en terotoxigenic E. coli isolated from humans, as well as non-toxigenic strains, normal fecal isolates of E. coli, and enterotoxigenic E. coli strains isolated from animals. This study has been published in Infection and Immunity, 32:1254-1260, 1981.

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Deneke, C.F., Thorne, G.M. and S.L. Gorbach. Attachment pili from enterotoxigenic Escherichia coli pathogenic for humans. Infect. Immun. 26:362-368, 1979.

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1. Detection of adherence antigens (attachment pili) associated with recent ETEC isolates from various geographic sources.

During the Contract period, we have continued to screen Escherichia coli isolated from humans with diarrhea for their ability to produce LT and/or ST enterotoxins and for the presence of surface antigen(s) analogous to the K88 antigen of porcine enteropathic E. coli. An outline of the characteristics of known adherence surface antigens (pili) found on ETEC strains of human origin is given in Table 1.

The Evans group have described antigen CFA/I and CFA/II. Production of these two antigens always correlates with the appearance of fine filamentous projections covering the surface of the bacterial cell (pili) and mannose-resistant hemagglutination activity for specific rbc types. These two antigens have been found on ETEC of a limited number of O-groups, mostly in the 078 serogroup in the case of CFA/I. These investigators have used an infant rabbit intestinal colonization assay as their adherence model.

Our studies provide evidence for at least 3 serologically distinct groups of surface antigens present on ETEC isolated from man. E. coli strains in each of the 3 serologic groups produce surface pili of identical size, mophology, and molecular weight. Antisera against these three pili types were used to test 106 strains of eneterotoxigenic E. coli isolated from humans, as well as non-toxigenic strains, normal fecal isolates of E. coli, and enterotoxigenic E. coli strains isolated from animals. This study has been published in Infection and Immunity, 32:1254-1260, 1981. The findings are outlined below.

A. Number of reactive strains. Of the 106 ETEC strains examined, 60 (56%) reacted with one or more of the antisera developed against the three pili types. The remaining 46 ETEC strains were nonreactive in these three antisera (Table 2). One strain, H10407, reacted with a high titer (1:4,096) with antisera against the pili of both strains 334 and D542. Five strains reacted with a titer of 1:1,024 or greater with antisera against pili of strains 334 and M9800-5. These six strains are listed in Table 2, as 1, 3 and 2, 3 reactive strains, respectively. The antisera were nonreactive with the 24 strains of nontoxigenic E. coli isolated from healthy adults, the rabbit pathogenic strains RDEC-1, and the porcine pathogenic strain P307 (K88+), as well as laboratory derivatives producing K88 or K99 antigen (Table 3).

B. Geographic distribution. The geographic sources of these strains and their reactivity with the pili antisera is given in Table 4. Whereas clustering of one pili serotype within a given outbreak was noted (e.g., all three stains from the Crater Lake outbreak were reactive with pili serogroup 2), there did not seem to be an association between pili serogroup and locale.

C. Enterotoxins. The toxin profiles of these 106 strans are recorded in Table 5. Again, there seemed to be no pattern of toxin production and pili serogroup.

D. Serotype. The distribution of O antigens within each of the pili serogroups is give in Table 6. No absolute correlation between pili

serogroups and 9 antigenic groups was observed, although there was some tendency for group 1 (334-like) strains to be O15 (57%) and for group 2 (M9800-5-like) to be O6 (32%). However, no pili group included only a single somatic antigenic type, and each somatic serotype occurred in at least two pili reactive groups.

E. MR-HA. The MR-HA patterns are shown in Table 7. Of the 106 strains, 38 (36%) were reactive with at least one species of the erythrocytes tested. The MR-HA reaction detected fewer ETEC strains than did the pili antisera, which reacted with 60 (56%) of the strains. The only discernible pattern is that strains which showed MR-HA with human A erythrocytes also reacted with human B erythrocytes. Of those strains which did react with erythrocytes, there was no pattern of pili serogroups.

2. Electron microscopic studies, isolation and purification and serologic characterization of "new" adherence antigens (pili).

We have been immunizing rabbits with isolated pili preparations harvested from three ETEC strains which fail to react with the three serologic antisera, but which undergo MR-HA reactionss and are piliated as detected by electron microscopic study. Strains which are currently under study or will be in the future, are described in detail in Table 8. These strains most likely possess a yet unclassified pili.

Bacterial agglutination reciprocal titers using adherence pili specific antisera are given in Table 9. High specificity for bacterial agglutination

was seen when the three prototype piliated ETEC strains were reacted with their respective pili antisera. Only limited cross-reactivity between these organisms and the heterologous antisera was detected. This cross-reactivity was of the same order of magnitude as the reactivity with normal (preimmunization) rabbit sera. Three ETEC strains with unclassified pili were also tested. These strains are MR-HA positive and have pili by electron microscopy. Strains H326c1 and 70-5206 are unreactive in the three pili antisera and presumably produce a serologically distinct pilus type (s). Strain 2016-10 reacts most strongly with its homologous sera but does show a significant but lower titer with type 3 antisera. Likewise the strain D542 (the type 3 pili prototype strain) also reacts with 2016-10 pili antisera. These two strains may be producing serologically related pili. The reactions of ETEC strain H10407 (CFA/I+) are discussed in the following section. As new pili antisera become available, Ouchterlony immunodiffusion analysis wae used to determine the number of antigenically reactive species which are being produced.

3. Serologic relationship of adherence antigen types 1, 2, 3 and CFA/I and CFA/II.

In our Ouchterlony immunodiffusion analysis of isolated pili, we have been able to detect three serologically distinct types of adherence pili. Our type 1 strain 334 (O15:H11) causes MR-HA of human A, B, bovine and guinea pig red cells. The type 2 prototype M9800-5 (O6:K15:H16) only causes MR-HA of bovine red cells. The type 3 prototype D542 (not typable) causes MR-HA of both human A and bovine red cells. Following isolation by our

red-cell absorption-elution techniques, all three pili types were found to have subunits with molecular weights of 12,500 and 13,100 by SDS PAGE (1). Thus, according to published data, these pili differ from CFA/I antigen of 23,800 molecular weight reported by the Evans' (Table 1) (2). Alternatively, we may be reporting the monomer size while the Evans' report a dimer configuration. In support of this possibility, Wevers et al, (3) has reported the molecular weights of subunits of CFA/I and CFA/II as 12,000 and 13,000 respectively, when using SDS-PAGE analysis (Table 1).

The CFA/I strain, H10407, reacts with both our type 1 and 3 antisera, and this has raised concern about the specificity of the pili specific antisera (Table 9). We prepared CFA/I by the Evans' method (2) and ran Ouchterlony immunodiffusion tests with type 1, 3 pili antisera. The results (single, crossed immunoprecipitin lines) indicate that the CFA/I preparation contained both our type 1 and 3 pili antigens but that these antigens are not identical. Further analysis with CFA/I antisera (from Dr. M. Levine) showed non-identity between CFA/I and our group 1 antigen but partial identity with group 3 antigen. At present we cannot explain all the variations of these immunologic reactions but they are undoubtedly affected by the particular strain of H10407 used and by the two different methods of antigen isolation (1,2).

4. Specificity of antisera against adherence antigens type 1, 2, 3.

Isolated pili preparations used to produce our three types of antisera have been subjected to electron microscopic examination, and piliate

structures were indeed seen in these preparations. One would need monoclonal antibody preparations to be sure that the sera are reacting with adherence antigens only. However, a reasonable level of confidence in the specificity in the antisera can be reached because of the following reasons:

(1) the antisera are prepared against purified pili, using a red cell absorbed-eluted technique.

(2) the antigens give two subunit bands in SDS-PAGE without foreign proteins.

(3) EM examination shows only pili structures.

(4) Ouchterlony immunodiffusion testing with isolated pili and pili specific antiserum gives single precipitin bands.

In our screening of 106 ETEC strains form around the world, other strains were found which, like H10407, reacted with one or more of our three pili antisera. Presumably, such strains are producing more than one serologic type of pilus.

In summary, the differences between our three pili types and the Evans' CFA/I and II are evident in both the comparison of the published data and in our serologic testing to date.These findings indicate the necessity for more studies in order to clarify the relationships of these important bacterial antigens. A completed biochemical (SDS-PAGE analysis) and serologic characterization of these antigens (prepared by both methods) is nearing completion.

5. Screening human ETEC strains with CFA-antisera and type 1, 2, and 3 adherence pili antisera.

Evans et al (4) have reported detecting CFA/I and CFA/II on 65 of 179 ETEC strains (36.3%). Orskov and Orskov (5) found 16 of 77 ETEC strains (20.8%) reacted with CFA/I antisera while Gross et al (6) found only 6 of 90 strains (6.7%) reacted with their anti CFA/I sera. In contrast, using the battery of 3 human attachment pili antisera we have demonstrated reactivity with 60 of 106 ETEC strains (56%). As well as detecting a greater percentage of human ETEC strains, our antisera also detects adherence pili on ETEC strains which produce LT/ST, LT-only and ST-only. The relative detection percentages of strains with these 3 toxin profiles was 75.6, 33.3 and 59.2%, respectively. In contrast, the CFA system detects primarily LT/ST strains detecting only 17.3% of LT-only and 70% ST-only strains (7). Thus, our attachment pili antisera react with a greater percentage of human ETEC strains and with a wider toxin profile than the CFA system.

6. Development of adherence assay using human ileal cells.

We believe that it is important to use human cells to study the attachment of bacterial strains pathogenic for man. Buccal cells or red blood cells are not the most appropriate target cell. Therefore, we have made an effort to prepare human small intestinal cells in order to have a better model for adherence studies. We have established a protocol for the use of intestinal cells from ileostomy patients.

Ileal cell fractions were diluted in buffer 2 to a concentration of approximately 10^4 to 10^5 ileal cells/ml. Cell counts were made using a hemocytometer. The number of viable ileal cells in the preparation was determined by trypan blue staining. There was considerable variation in both the absolute cell yield and the composition of the ileal cells from two-thirds of experiments. Of those preparations that yielded adequate cells, the inital lavage fluid contained approximately 85% viable cells. This number decreased to 70% in the ileal cell fraction.

A. Binding of ETEC strains to human ileal cells. A Nuclepore filatration-binding assay was used, similar to that described for ETEC binding to huma buccal mucosal cells. Five different, human pathogenic ETEC strains bound to human intestinal cells to a greater extent ($\geq 4.8 \times 10^6$ bacteria/filter) than did the $K99^+$ producing animal pathogens, strain RDEC-1, an ovine pathogen, and non-toxinogenic control strain, 334LL ($\leq 0.6 \times 10^6$ bacteria/filter). The binding was reproducible over several sets of experiments. The K88 pili antigen producing strains have consistently bound to the human ileal cells throughout this study, to an extent similar to that of the human pathogenic strains. The mechanism of K88 binding to human cells is not clear but could reflect the presence of a receptor on the human ileal cells similar to the K88 receptor of animal intestinal cells. Additional K88 strains are being investigated.

7. Genetic studies of plasmids controlling production of adherence antigens.

During the past year, we have attempted to clone DNA controlling pili production onto plasmid pBR322 using restriction-ligation techniques. This relaxed plasmid, 2.6 x 10^6 daltons, contains a unique PstI site within an ampicillin-resistane gene and four other unique restriction sties (EcoR1, Hind III, BAm H1 and Sal I) in the tetracycline-resistance gene.

This plasmid is sensitive to col E1, and the antibiotic resistance genes on pBR322 are not transposable. Briefly, preparations of vector plasmid DNA (pBR322) and plasmid DNA from a pili-producing ETEC strain are treated with a single restriction enzyme. At the present time, we are employing Bam H1, although with this vector four other restriction endonucleases can be used. Bam H1 was chosen because it is known to cleave outside the K99 region (S. Falkow, personal communication). Restriction enzyme cleavage products are then incubated with T_4 DNA ligase, and used to transform E. coli recipient strain JE2571 (pil, fla, str, leu, thr), a strain which does not produce flagella or pili, is streptomycin resistant, and requires leucine and threonine for growth.

In experiments, transformed colonies were selected on ampicillin (25 μg/ml) containing medium and screened for tetracycline sensitive clones which would result from insertion of cloned DNA into the Bam H1 site. A piliated transformant was isolated from an experiment using ligation products of Bam H1 treated pBR322 the vector plasmid. This Amp^r, Tc^s, transformant JE(pGT542) has identical phenotypic markers as the parental

strain JE2571. The transformant however was found to cause a weak MR-HA reaction with human type A blood. Upon electron microscopic study, fine piliate structures identical in size to those of the parental ETEC strain D542. Pili isolated from the transformant have been found to react with antisera against pili from strain D542 in ouchterlony immunodffusion assays.

The hybrid plasmid has a molecular size approximately 6.4 x 10^6 daltons as calculated from analysis of CCC moleclues. Bgl I treatment of the hybrid and vector plasmids results in release of 2 fragments and from the size of these one could estimate that a 3.6 - 4.0 M dalton piece has been inserted in the hybrid plasmid. Restriction mapping of the hybrid plasmid and sub-cloning experiments are planned in order to further isolate and characterize the genes involved in production of these adherence pili.

The hybrid plasmid has been transformed into several E. coli strains: 334LL, the plasmid-free non-toxigenic, MR-HA$^-$ derivative of ETEC 334, and CD-1, a plasmid-free non-toxigenic MR-HA$^-$ fecal isolate. Tests for LT enterotoxin production by these strains were negative in the Y1 adrenal tissue culture assay and negative for ST production in the suckling mouse assay. The hybrid plasmid containing strain will shortly be tested for adherence to human buccal cells and human ileal cells, and undergo further serologic classification of its pili by ElISA techniques. Long range plans include cloning the pili genes from protype ETEC strains producing serologically different pili and comparing these pili genes and plasmids for areas of DNA homology.

Overall, the studies described above allow for better definition of intestinal adherence factors and thus ultimately lead to better control of ETEC diarrhea.

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TABLE 1

ETEC ADHERENCE ANTIGENS

TYPE:	ADHERENCE PILI	CFA/I	CFA/II
MR-HA	Serogroup 1,2,3		
RBC Type	Human A, B+	Human A^+	Bovine$^+$
	Bovine $\pm$	Bovine$^+$	
	Guinea pig $\pm$	Chicken$^+$	
MOLECULAR:	13,100	23,800	
	12,500	(12,000)*	(13,000)*
SIZE:	5-10nm	7nm	
TISSUE:	Human buccal mucosa Human ileal cells	Rabbit intestine	Rabbit intestine
ENTEROTOXIN(S)	LT/ST ST^+, LT^+	LT/ST ST^+	LT/ST
O-Group:	06, 08, 015, 020	06, 08, 015	06, 09
	025, 027, 078	025, 078	085
	0128, 0148, 0149		

*Wevers et al, 1980 Characterization of pili associated with Escherichia coli 018ac Infect. Immun. 29:685-691

TABLE 2. Reactivity of 106 ETEC Strains of Human Origin With Antipili Sera[a]

Serogroup	No. of reactive strains (%)
1	12 (11.3)
2	34 (32.1)
3	8 (7.5)
1,3	1 (0.9)
2,3	5 (4.7)
Negative (nonreactive)	46 (43.4)

[a]ETEC strains obtained from throughout the world were titered with antisera prepared against MR-HA pili of strain 334 (serogroup 1), strain M9800-5 (serogroup 2), and strain D542 (serogroup 3).

TABLE 3.

E. Coli Control Strains

Animal Origin	Serotype	MR-HA[1] AB	GP	BOV	Pili Serogroups
RDEC-1 (Bovine diarrhea)	?	-	-	-	NR
P307 (Porcine Diarrhea)	-K88^{+}	-	+	-	NR
Lab Derived					
K 12					NT
K 12 K88	K88	-	+	-	NT
K 12 K99	K99	-	-	-	NT
Adult Feces (Non-Toxigenic)					
H10405 (LT ? Klipstein)	?	-	-	-	NR
HS	?	-	-	-	NR
CD-1	?	-	-	-	NR
Ec#1	?	-	-	-	NR
#7	?	-	-	-	NR
#8	?	-	-	-	NR
#9	?	-	-	-	NR
#10	?	-	-	-	NR
#11	?	+			NR
#12	?	-	-		NR
#13	?	+			NR
#14	?	-	-		NR
#15	?	-	-		NR
#16	?	-	-		NR

Table 3 (continued)

Animal Origin	Serotype	MR-HA[1] AB	GP	BOV	Pili Serogroups[2]
Adult Feces (cont'd)					
#17	?	+			NR
#18	?	-	-		NR
#19	?	-	-		NR
#20	?	+			NR
#21	?	-	-		NR

1. Mannose-resistant hemagglutination of washed human A, B, guinea pig and bovine RBC's at $4^{o}C$.

2. NR - non-reactive in antisera

   NT - not typable due to auto-agglutination.

TABLE 4.

Geographic Source of ETEC Strains

by Pili Serotype

Pili Serotype	Source (no. of isolates)
1	India (2), Dacca (2), Pakistan (1), United States (5), unknown (2)
2	U.S. Army (2), foreign 2), United States (6), Kenya (5), Dacca (4), Sweden (2), Ethiopia (2), Honduras (2), Vietnam (1), Mexico (2), unknown (6)
3	Dacca (3), Mexico (2), Honduras (2), Ethiopia (1)
1,3	India (1)
2,3	Texas (2), Dacca (3)
Negative	Pakistan (1), United States (3), Kenya (5), Vietnam (2), Morocco (2), Mexico (6), Ethiopia (4), Honduras (11), cruise ships (2), unknown (10)

TABLE 5.

Enterotoxin profile of *E. coli* Strains by Pili Serogroups

Pili Serogroup	No. strains producing toxin			
	LT and ST	LT	ST	ND[a]
1	6	3	3	0
2	20	4	8	2
3	4	1	3	0
1,3	1	0	0	0
2,3	3	0	2	0
Negative	11	16	11	8

[a]ND. Toxin profile either not reported or determined by nonstandard methods.

TABLE 6. Somatic O antigens of 106 ETEC Strains by Pili Serogroup

Pili serogroup	O antigens (no. of strains)
1	O15(8), O78 (1), O128 (2), ND[a] (1)
2	O6(11), O8 (1), O20 (1), O25 (1), O27 (3), O78 (1), O148 (1), ND (14)
3	O6 (1), O78 (1), O128 (1), ND (5)
1,3	O78 (1)
2,3	O78 (2), ND (3)
Negative	O6 (1), O15 (1), O25 (4) O27 (2) O78 (5), O128 (3), O148 (2), ND (28)

[a]ND, O antigen not determined.

TABLE 7. MR-HA of 106 ETEC strains by pili serotype

Pili serotype	MR-HA with erythrocytes[a]			No. of strains
	Human A and B	Bovine	Guinea	
1	+	+	+	4[b]
	+	-	+	2
	-	-	-	6
2	-	+	-	4[b]
	+	-	-	2
	-	-	-	23
	-	-	+	3
	+	+	-	
3	+	+	-	5[b]
	+	-	-	2
	-	-	-	1
1,3	+	+	-	1
2,3	+	+	-	4
	-	-	+	1
Negative	-	-	-	40
	+	+	-	2
	+	-	+	1
	+	-	-	3

[a]MR-HA was determined at 0°C using 1% mannose and washed erythrocytes.

[b]Includes prototype strain.

TABLE 8.

Enterotoxigenic E. coli

Possessing "novel" Pili Types

ETEC	Serotype	Toxins	MR-HA[1]	EM[2]	Origin
H326c1	Not tested	LT/ST	AB+ Bov+	+	Honduras
CDC5206-70	O128:H21	ST	AB+ GP+	+	CDC-New Mexico
54c14	078:KN:KM	LT/ST	AB+	+	Ethiopia
H410c1	Not tested	LT/ST	AB+ Bov+	+	Honduras
9b-8	078:KN:KM	LT/ST	AB+	+	Ethiopia
2016-10	Non-typable	ST+	AB+	+	Ethiopia
A40	Not tested	LT/ST	GP+	+	Panama
Pc-61d	Not tested	LT/ST	Bov+ GP+	+	Panama

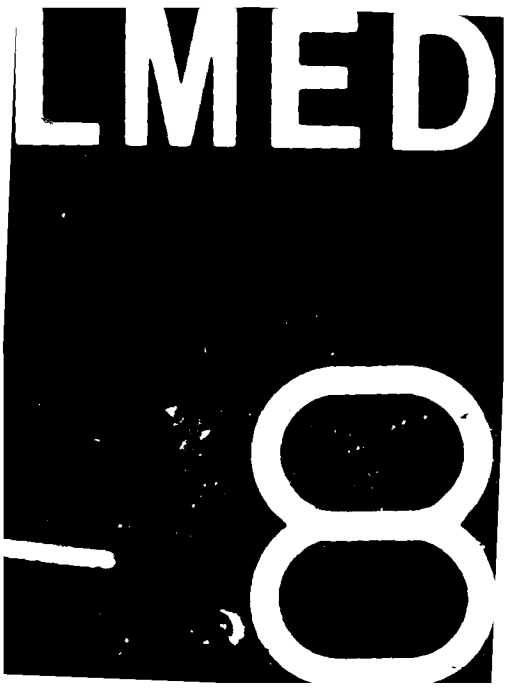
[1]Mannose-resistant hemagglutination reactions are performed at 4°C in the presence of 1% mannose using either human type A or B (AB+) bovine (bov+) or guinea pig (GP+) washed erythrocytes.

[2]Electron microscopic (EM) detection of pili 5-10mn in size following negative staining with 1% phosphotungstic acid.

TABLE 9

Bacterial Agglutination Reciprocal Titers Using
Adherence Pili Specific Antisera

Antisera	ETEC STRAINS						
	334 (type 1 pili)	M98005 (type 2 pili)	D542 (type 3 pili)	2016-10 (type ? pili)	H326c1 (type ? pili)	70-5206 (type ? pili)	H10407 (CFA/I)
Type 1	2048	64	128	64	64	64	2048
Type 2	256	4096	128	128	128	128	128
Type 3	128	128	2048	512	128	64	1024
2016-10 pili antisera	64	64	512	8192	64	128	2048
Normal rabbit sera	256	32	128	64	128	128	64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